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Brief Communication

INFLUENZA VIRUS INDUCES EXPRESSION OF ANTIOXIDANT GENES IN HUMAN EPITHELIAL CELLS

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Abstract—Influenza infections cause airway epithelial inflammation and oxidant-mediated damage. In this setting, cellular antioxidant enzymes may protect airway epithelial cells against damage resulting from toxic oxygen radicals produced by activated leukocytes. Therefore, we tested the effect of influenza virus infection, as well as exposure to human recombinant interferon-γ (IFN-γ), on gene expression for the antioxidant enzymes manganese supeoxide dismutase (MnSOD), copper/zinc superoxide dismutase (Cu/ZnSOD), indoleamine 2,3-dioxygenase (IDO), and catalase in primary cultures of human airway epithelial cells. In these cells, both viral infection and IFN-γ increased MnSOD and IDO mRNAs. In contrast, neither viral infection nor IFN-γ affected Cu/ZnSOD gene expression, and both viral infection and IFN-γ decreased catalase gene expression. The differential effects of viral infection on antioxidant gene expression and their further amplification by IFN-γ are likely to be important protective mechanisms in viral airway infections.

Keywords—Influenza, Virus, Antioxidants, Gene expression, Epithelium, Interferon, Superoxide dismutase, Indoleamine dioxygenase, Free radicals

INTRODUCTION

The epithelium of the lung and airways are the primary targets of influenza virus infections. Influenza infections of the airways lead to inflammation that is most intense in the epithelial layer.[1] This may be due to the fact that virus-infected airway epithelial cells synthesize and release interleukin-8,[2] a strongly chemotactic cytokine that also increases leukocyte oxygen radical production.

Production of oxygen radicals in the lung may contribute to virus-induced lung damage. In support of this relationship is the observation that the administration of antioxidants markedly decreases both lung damage and mortality in influenza-infected mice.[3,4]

A variety of enzymes contribute to cellular antioxidant defense. The detoxifiction of superoxide $O_2^{\cdot-}$ via the dismutation reaction: $O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ = O_2 + H_2O_2$, is catalysed by two structurally distinct superoxide dimutases. Copper, zinc superoxide dismutase (Cu/ZnSOD) is primarily a cytoplasmic enzyme that is constitutively expressed. Manganese superoxide dismutase (MnSOD) is located primarily in the mitochondria, and can be induced by oxidant stress as well as by a variety of cytokines.[5]

The dismutation reaction catalysed by these two enzymes yields the potentially toxic product hydrogen peroxide. Catalase may therefore also contribute to antioxidant defense by metabolizing hydrogen peroxide. Alternatively, superoxide can be detoxified by indoleamine dioxygenase (IDO), which uses superoxide to oxidize tryptophan.[6] Elimination of superoxide by this reaction does not produce toxic products.

Another aspect of virus-induced epithelial inflammation is release, by lymphocytes, of interferon-γ (IFN-γ), a cytokine with effects on both cellular gene expression and viral replication. The influx of lymphocytes into the airways may accompany the influx of superoxide-producing neutrophils and monocytes. Therefore, we investigated the effects of both influenza infection and exposure to IFN-γ on MnSOD, Cu/ZnSOD, IDO, and catalase gene expression in primary cultures of human airway epithelial cells.

MATERIALS AND METHODS

Cell culture

Human airway epithelium was cultured using a modification of the technique devised for dog airway

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epithelium by Coleman et al.[7] Main bronchi and lobar or segmental bronchi were obtained from surgical lung resections. Airways were left overnight at 4°C in a solution of 0.5% pronase in Hank's buffered salt solution (HBSS). Epithelial cells were removed from the underlying tissues by jets of 10% fetal bovine serum in HBSS. Cells were plated in LHC-8e medium (Biofluids)[8] on rat-tail collagen-coated six-well plates at a density of 1.2×10^5 cells/cm^2. The medium was changed every other day, and the cells grew to confluence in about one week.

Virus stock

Human influenza type A/Port Chalmers/72 (H3N2) (American Type Culture Collection) was grown in rhesus monkey kidney cell monolayers (Viromed). Cultures were then frozen and thawed to disrupt cells, and the infected culture fluid was cleared by centrifugation and stored in aliquots at −70°C. Uninfected monolayers were treated in a similar way to produce control medium.

To determine the viral content of these fluids, fresh kidney cell monolayers were exposed to serial 10-fold dilutions of this solution and incubated for one week. Viral infection of these cells was demonstrated by the ability to adsorb guinea pig erythrocytes, a phenomenon that reflects expression of viral hemagglutinin on the infected cell's surface. The dilution that infected 50% of cultures (the $TCID_{50}$) was determined as previously described.[9] The virus stock was found to contain 10^7 $TCID_{50}$ per ml. No virus was detected in the control medium.

As this is a human influenza virus, there was the potential for infection of laboratory personnel. All viral work was done in a tissue culture hood by personnel wearing gloves. Work areas were cleaned using a 70% ethanol solution. Similar care was taken in the handling of human airway tissues.

Viral infection of epithelial cells

As previously described,[10] medium was removed from confluent airway epithelial cell monolayers and was replaced with either virus stock diluted in LHC-8e medium to a concentration of 10^5 $TCID_{50}$/ml or control medium similarly diluted. After 1 h, the medium was replaced with fresh LHC-8e. The medium was again changed 24 h later, and was collected 48 h after infection. RNA was isolated from the cells at 48 h. Viral infection of airway epithelium was demonstrated by recovering virus from the culture medium into fresh monolayers of Rhesus monkey kidney cells as described above. A hemadsorption assay was also performed in

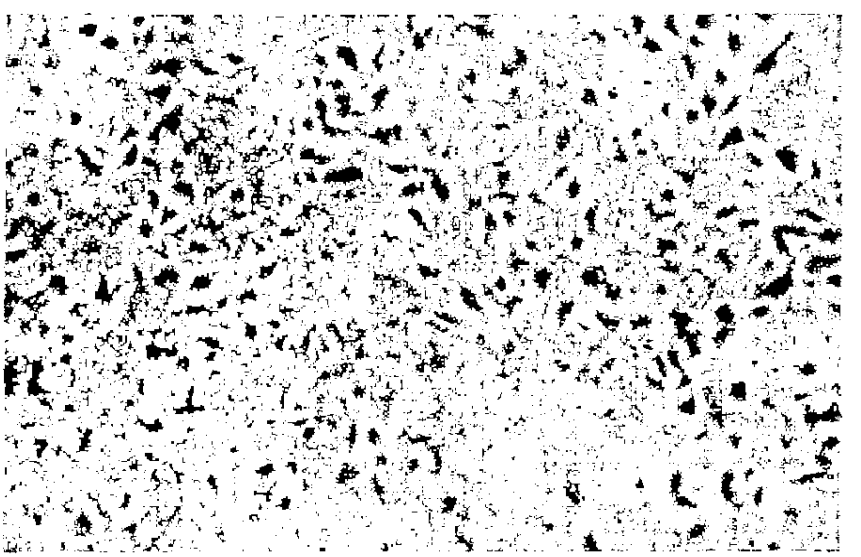

Fig. 1. Virus-infected human airway epithelial cells (dark polygonal cells) encrusted with guinea pig erythrocytes (overlying the monolayer in areas where the epithelial cells are not visible). Forty-eight h after infection, epithelial monolayers were washed and overlaid with a 0.5% suspension of guinea pig erythrocytes. After 30 min at 4°C, monolayers were again washed to remove nonadherent erythrocytes. Adsorption of erythrocytes reflects epithelial cell expression of influenza hemagglutinin. Phase microscopy (original magnification 400×).

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one airway epithelial monolayer from each experiment to demonstrate that the cells were infected.

Treatment with IFN-γ

For IFN-γ treatment, the medium was removed from confluent monolayers and replaced with LHC-8e medium that contained human recombinant IFN-γ (300 U/ml). RNA was isolated from human airway epithelial cells after 48 h.

RNA isolation and northern blot analysis

Total RNA was isolated by the RNAzol B method with direct lysis in RNAzol lysis buffer followed by chloroform and phenol/chloroform extractions (Cinna/Biotex). Northern blot analysis was performed as previously described[11] with hybridization and washing conditions carried out as described by Church and Gilbert.[12] To control for variation in either the amount of RNA in different samples or loading errors, all blots were rehybridized with an oligonucleotide probe (5′ ACG GTA TCT GAT CGT CTT CGA ACC 3′) corresponding to 18s RNA after stripping of the original probe. Ethidium bromide staining of RNA samples were also performed to asses integrity of RNA and equal loading of RNA. Autoradiograms were quantitated by densitometric scanning. Densitometric values were normalized to values obtained for 18s RNA obtained on the same blot.

Probes

Rat MnSOD and Cu/ZnSOD cDNAs were obtained from Dr. Harry Hick (University of Florida, Gainesville, FL). Human catalase cDNA was provided by Dr. Steven Akman (City of Hope National Medical Center, Duarte, CA). A 39 mer oligonucleotide (5′ GGT GAT GCA TCC CAG AAC TAG ACG TGC AAG GCG CTG TGA 3′) complementary to human IDO was synthesized using an ABI 391 DNA synthesizer (Applied Biosystems Inc., Foster City, CA).

RESULTS

Viral infection of epithelial cells

Primary cultures of human airway epithelial cells became infected as reflected by the ability of monolayers to adsorb guinea pig erythrocytes (Fig. 1), as well as by the recovery of infectious progeny virus from the supernatant culture medium (data not shown).

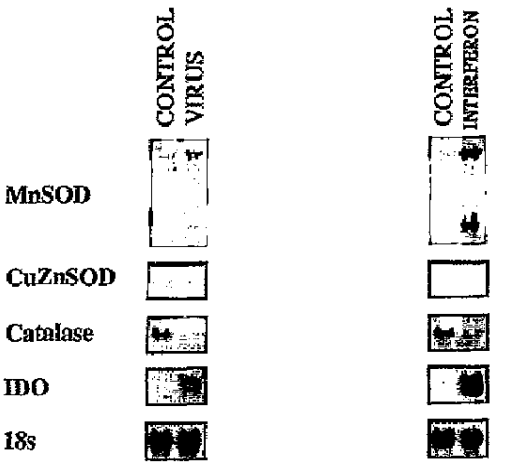

Fig. 2. Northern blot analysis of RNA extracted from control monolayers, virus-infected monolayers, and monolayers treated with IFN-γ 300 units/ml for 48 h. Blots were probed for each of the anitoxidants listed, then with a probe for 18S ribosomal RNA as a control for variation in RNA loading. Blots are representative of three experiments.

Effects of viral infection and IFN-γ on antioxidant gene expression in primary cultures

As shown in Figure 2, 48 h after infection of primary airway epithelial cell cultures, MnSOD and IDO mRNA were increased 3–4-fold. Exposure of uninfected cells to IFN-γ for 48 h increased both MnSOD and IDO mRNA by more than 20-fold. In contrast, neither viral infection nor treatment with IFN-γ affected Cu/ZnSOD mRNA, while catalase mRNA decreased after both infection and treatment with IFN-γ.

DISCUSSION

These data show that both influenza virus infection and IFN-γ induce MnSOD and IDO gene expression in human airway epitheial cells. As pulmonary epithelium is the primary site of influenza infection, and because such infections cause inflammation of the epitheial layer, virus-infected epitheial cells are likely to be exposed to $O_2^{\cdot-}$ produced by activated leukocytes, as well as from other sources. Increased MnSOD and IDO activity in this setting may protect the epitheium against oxidant damage. IFN-γ-induced expression of MnSOD and IDO mRNA in primary cultures of human airway epithelium confirms previous reports of IFN-γ-induced expression of these genes in A549 and HEp-2 neoplastic epithelial cell lines,[13,14] and suggests a mechanism for further increases in antioxidant defenses in the face of virus-induced airway inflamma-

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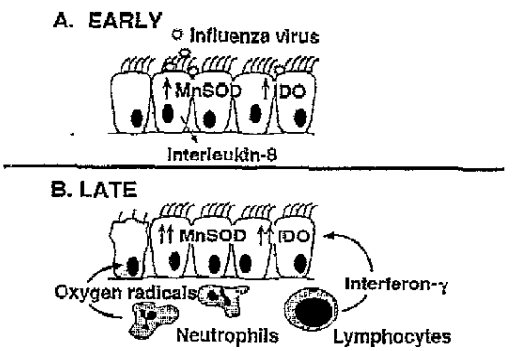

Fig. 3. Events leading to epithelial inflammation and protection of epithelial cells against oxidant injury. (A) Early changes accompanying influenza infection: epithelial cells increase expression of MnSOD and IDO, and release interleukin-8, which promotes inflammation. (B) Late changes after infection: leukocytes recruited and activated by epithelial interleukin-8 produce toxic oxygen radicals, but at the same time release IFN-γ, which further increases expression of MnSOD and IDO, thereby augmenting antioxidant defenses.

tion. Furthermore, in vivo influenza infection in mice induces expression of both MnSOD (Jacoby and Choi, unpublished observations) and IDO.[15]

Oxidant damage has been shown to contribute to the pathogenesis of influenza-induced lung damage in mice.[3,4] Superoxide production both by activated neutrophils, as well as that resulting from increased xanthine oxidase activity in the lung, may be important in producing oxidant damage in virus-infected lungs.[3,4]

These data provide the first evidence of a direct effect of viral infection on cellular antioxidant gene expression. Induction of MnSOD and IDO by virus infection in epithelial cells may be an important defense against oxygen radical-mediated damage. Our findings concerning the effects of influenza virus infection on airway epithelial cell gene expression suggest the scenario depicted in Figure 3. Viral infection induces expression of interleukin-8,[2] which promotes both the influx and the activation of inflammatory cells. At the same time, viral infection induces expression of the antioxidant enzymes MnSOD and IDO as early as 48 h after infection, a time that is likely before the invasion of the airway by inflammatory cells.[16] In a sense, this prepares the epithelial cell for the oxidant stress that results subsequently from the inflammatory cells recruited and activated by epithelial interleukin-8. Release of IFN-γ by activated lymphocytes in the virus-infected lung and airways will further induce MnSOD and IDO gene expression, providing further protection against oxidant stress.

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ABBREVIATIONS

Cu/ZnSOD—copper/zinc superoxide dismutase
IDO—indoleamine 2,3-dioxygenase
IFN-γ—interferon-γ
MnSOD—manganese superoxide dismutase

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